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Steroidogenesis in the Ovarian Follicles of the Medaka (*Oryzias latipes*) during Vitellogenesis and Oocyte Maturation

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ABSTRACT—Changes in the steroidogenic pathway in medaka (*Oryzias latipes*) ovarian follicles during vitellogenesis and oocyte maturation were investigated *in vitro* by incubation of follicles with several radiolabeled steroid precursors, followed by thin layer chromatography (TLC) fractionation and recrystallization. When vitellogenic follicles collected at 18 hr before the expected time of spawning were incubated with ^{3}H-labeled pregnenolone, the major metabolites were 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and androstenedione. Incubations of vitellogenic follicles with androstenedione produced testosterone and estradiol-17β. By contrast, when maturing follicles (postvitellogenic follicles undergoing maturation) collected at 10 hr before spawning were incubated with ^{3}H-labeled pregnenolone, the major metabolites were 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP, maturation-inducing hormone of medaka); androstenedione was not detected. Neither vitellogenic and maturing follicles produced progesterone when they were incubated with ^{3}H-labeled pregnenolone, suggesting that in medaka ovarian follicles both estradiol-17β and 17α, 20β-DP are synthesized by the ^{5}Δ-steroid pathway. Thus, there is a distinct shift in the steroidogenic pathway from estradiol-17β to 17α, 20β-DP production in medaka ovarian follicles, and it is suggested that the decrease in C17, 20-lyase activity is responsible for this shift.

The phosphodiesterase inhibitor IBMX enhanced androstenedione production in incubations of vitellogenic follicles with ^{14}C-labeled progesterone. Calcium ionophore A23187 and the phorbol ester TPA (a protein kinase C activator) blocked the stimulatory actions of IBMX on androstenedione production. These findings suggest that multiple signalling pathways may participate in the regulation of ovarian steroidogenesis, and further emphasize the importance of calcium as a regulator of P-450c17 activity.

INTRODUCTION

In nonmammlian vertebrates, ovarian follicle cells produce two different steroid hormones, estradiol-17β and maturation-inducing hormone (progestogens), in response to pituitary gonadotropins, which play important roles in two phases of oogenesis, vitellogenesis and oocyte maturation, respectively. Estradiol-17β promotes vitellogenesis in members of all non-mammalian vertebrates (Ho, 1991). In each of these groups, the time of vitellogenin production corresponds to the period of elevated plasma estradiol-17β levels. Vitellogenin is the precursor molecule for egg yolk, which is of considerable importance as the source of metabolic energy for the developing embryo. In response to increased levels of plasma estradiol-17β, the liver synthesizes and secretes vitellogenin, which is carried in the bloodstream to the oocytes. The developing oocytes take up vitellogenin and convert it to egg yolk. On the other hand, a variety of progestogens have been shown to be effective in the initiation of meiotic maturation in fish and amphibian oocytes (Nagahama, 1987). Two progestogens, 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) and 17α, 20β, 21-trihydroxy-4-pregnen-3-one have been identified as the maturation-inducing hormone. These ovarian steroid hormones are known to be secreted from ovarian follicles in response to pituitary gonadotropins. A distinct shift from estradiol-17β to 17α, 20β-DP (the maturation-inducing hormone of salmonid fishes) has been reported to occur in ovarian follicles immediately prior to oocyte maturation (Nagahama *et al.*, 1994), and seems to be a prerequisite step for growing oocytes to enter the final stage of maturation. However, the regulatory mechanism of this steroidogenic shift is not clearly understood.

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The medaka, *Oryzias latipes*, under a long photoperiod (14 hr light-10 hr dark) at 26°C, spawns daily within 1 hr of the onset of light for a number of consecutive days. Under these conditions, the sequence of events leading to spawning such as vitellogenesis, oocyte maturation, and ovulation can be timed accurately (Iwamatsu, 1978). These features make medaka an ideal model in which to investigate the regulatory mechanism of the steroidogenic shift occurring in ovarian follicles prior to oocyte maturation. We have previously shown that medaka ovarian follicles produce estradiol-17β during vitellogenesis and 17α, 20β-DP during oocyte maturation (Nagahama *et al.*, 1991; Sakai *et al.*, 1987, 1988). During vitellogenesis, cytochrome P-450 aromatase (P-450arom) activity is high, but 20β-hydroxysteroid dehydrogenase (20β-HSD, the enzyme which converts 17α-hydroxyprogesterone to 17α, 20β-DP) activity is nondetectable, leading to the production of estradiol-17β. By contrast, during oocyte maturation, P-450arom activity is low, but 20β-HSD is high, leading to the production of 17α, 20β-DP. 17α, 20β-DP was identified as a major maturation-inducing hormone in medaka (Fukada *et al.*, 1994). Thus, there is a shift in steroidogenic enzyme activity (a decrease in P-450arom activity and an increase in 20β-HSD activity) and this shift is a mechanism regulating the shift from estradiol-17β to 17α, 20β-DP production in medaka ovarian follicles. However, a shift in precursor production from testosterone to 17α-hydroxyprogesterone is also an important mechanism for the shift from estradiol-17β to 17α, 20β-DP production. To determine a key enzyme(s) responsible for this shift in steroid precursor production, the precise information on the steroidogenic pathway during vitellogenesis and oocyte maturation is required.

In this study, the steroidogenic pathway in medaka ovarian follicles during vitellogenesis and oocyte maturation was determined. To this end, intact follicles or follicle cell layers, isolated at appropriate time before spawning, were incubated with radiolabeled steroid precursors, and metabolites were analyzed by TLC fractionation and recrystallization. Since the conversion of 17α-hydroxyprogesterone into androstenedione is expected to be a key step of the shift in medaka ovarian steroidogenesis from vitellogenesis to oocyte maturation, we focused on the production of androstenedione. We also tested the effects of several reagents on the production of androstenedione *in vitro*.

MATERIAL AND METHODS

Chemicals

[7-^{3}H(N)]-pregnenolone (370-925 GBq/mmol), [1, 2-^{3}H]-17α-hydroxyprogesterone (1.48-2.22 TBq/mmol) and [4-^{14}C]-androstenedione (1.67-2.22 GBq/mmol) were obtained from NEN research products. These steroids were purified by TLC with appropriate solvent systems before use. Cold steroids, progesterone, androstenedione, 17α-hydroxyprogesterone, testosterone, and 17α, 20β-DP were obtained from Sigma. Pregnant mare serum gonadotropin (PMSG) was purchased from Teikoku Zohki (Tokyo, Japan). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthin (IBMX), calcium ionophore A23187, and the phorbol ester TPA were purchased from Sigma.

Ovarian follicle incubation

Medaka were obtained from a local fish farm (Yatomi, Aichi, Japan). Fish were killed by decapitation and ovaries were immediately removed and kept in cold modified Earl's medium 199 (Gibco BRL, containing 30 mg/liter streptomycin sulfate and penicillin G potassium. pH was adjusted with 25 mM Hepes-NaOH into 7.3, diluted into 90% of original concentration). Premature (including fully grown oocytes) and maturing (undergoing oocyte maturation) follicles were collected from several ovaries with the aid of fine forceps. In some experiments, follicle cell layers were obtained by squeezing out yolk from intact follicles. Isolated follicle cell layers or intact follicles were incubated in six well/flat bottom plastic cell wells containing 10 ml modified Earl's medium 199 with radiolabeled steroid precursors. In some experiments follicle cell layers or intact follicles were incubated in the presence of PMSG (100 units/ml) or other chemical reagents. Incubations were performed under humid conditions at 26°C.

Analysis of metabolites

Steroids were extracted from incubation media with 3 ml of dichloromethane five times and dried on warm sand bath under nitrogen gas. Dried extracted steroids were resuspended with a small volume of dichloromethane and applied on a TLC plate containing a fluorescent indicator (Merck). Mixture of progesterone, androstenedione, 17α-hydroxyprogesterone, testosterone, and 17α, 20β-DP was applied on a TLC plate with samples as standards of mobility. Standard steroids were detected by UV absorption at 254 nm. The plates were usually developed in benzene : acetone (4:1; v/v) and in some experiments in other solvent systems.

When ^{3}H-labeled steroids were used as precursors, autoradiography was carried out with ^{3}H-hyperfilms (Amersham). In case of ^{14}C-labeled precursors, results were analyzed with a BAS 2000 bio-imaging analyzer (FUJIFILM). Some of the bands were scraped and metabolites were extracted with dichloromethane. Extracted steroids were identified by recrystallization method. Recrystallization was performed in accord with Axelrod *et al.* (1965).

RESULTS

Incubations of vitellogenic and maturing follicles with [7-^{3}H(N)]-pregnenolone and [1,2-^{3}H]-17α-hydroxyprogesterone

Vitellogenic follicles collected at 18 hr before the expected time of spawning and maturing follicles (follicles undergoing maturation) collected at 10 hr were incubated with 5×10^{6} cpm of [7-^{3}H(N)]-pregnenolone for 3 hr and the steroid metabolites produced during incubations were separated by TLC-I, benzene-acetone (4:1). There was a marked difference in the pattern of radioactive steroid metabolites between vitellogenic and maturing follicles. The following steroid metabolites produced in vitellogenic follicle incubations were identified by their chromatographic mobilities in TLC: pregnenolone (precursor, metabolite 1), 17α-hydroxyprogesterone (metabolite 2), 17α-hydroxypregnenolone (metabolite 3), and androstenedione (metabolite 4) (Fig. 1A). The identities of the three metabolites as 17α-hydroxypregnenolone, 17α-hydroxyprogesterone and androstenedione were confirmed by recrystallization to constant specific activity (Table 1). When maturing follicles were incubated with ^{3}H-labeled pregnenolone, two major metabolites (17α-hydroxypregnenolone and 17α-hydroxyprogesterone) and two minor polar metabolites were found; however, no radioactivity comigrated with androstenedione (Fig. 1B).

Vitellogenic and maturing follicles were also incubated

Table 1. Specific activities of recrystallization

Steroid	Hours before spawning	Specific activities of crystals (cpm/mg)			
		1st	2nd	3rd	before crystallization
17α-Hydroxypregnenolone	10	25300	25700	22700	27200
17α-Hydroxypregnenolone	18	6150	6800	6480	7590
17α-Hydroxyprogesterone	10	170	167	169	195
17α-Hydroxyprogesterone	18	26.7	25.6	26.4	28.6
Androstenedione	18	15.4	14.8	14.6	17.1

with 5×10^6 cpm of [1,2-^{3}H]-17α-hydroxyprogesterone for 3 hr. Autoradiographs of the steroid metabolites show that 17α-hydroxyprogesterone was not extensively metabolized by either vitellogenic or postvitellogenic follicles. The yield of androstenedione was much higher in incubations of vitellogenic follicles than those of maturing follicles (Fig. 1C). Minor polar metabolites were found in incubations of maturing follicles (Fig. 1D).

Incubation of vitellogenic and maturing follicles with [4-^{14}C]-androstenedione

After chromatography of the vitellogenic follicle incubation with androstenedione gave several radioactive metabolites corresponding to carriers androstenedione (precursor, metabolite 1), testosterone (metabolite 2) and estradiol-17β (metabolite 3) (Fig. 2A). The testosterone area when rechromatographed in TLC-II, cyclohexane-ethanol (9:1), gave a major metabolite (metabolite 1) with the same mobility as the carrier standard testosterone (Fig. 3A). The estradiol-17β area when rechromatogaphed in TLC-II gave three metabolites: one of the metabolites was comigrated with estradiol-17β (Fig. 3B). Androstenedione was not extensively metabolized by maturing follicles with very few metabolites produced (Fig. 2B).

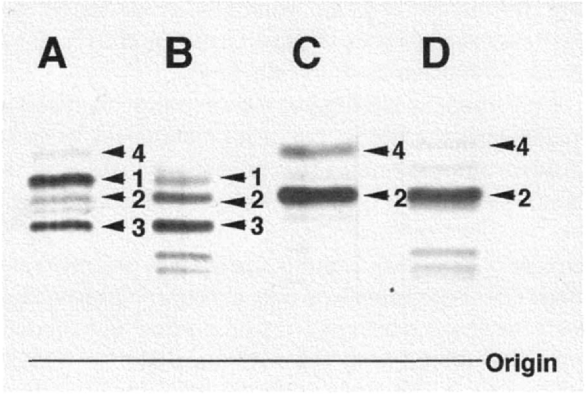

Fig. 1. Autoradiograms of steroid metabolites produced by medaka ovarian follicles incubated with [7-^{3}H(N)]-pregnenolone (A, B) or 17α-hydroxyprogesterone (C, D). Ovarian follicle cells were collected at 18 hr (A, C) or 10 hr (B, D) before spawning. 1, pregnenolone; 2, 17α-hydroxyprogesterone; 3, 17α-hydroxypregnenolone; 4, androstenedione.

Incubation of vitellogenic follicles with [1, 2-^{3}H]-17α-hydroxyprogesterone in the presence of PMSG

Medaka vitellogenic follicles collected at 22 hr before spawning do not undergo spontaneous maturation, but undergo maturation in response to 17α, 20β-DP (Iwamatsu, 1974, 1978). Intact follicles (200 follicles/well) were incubated in the presence or absence of PMSG for 2 and 12 hr. These

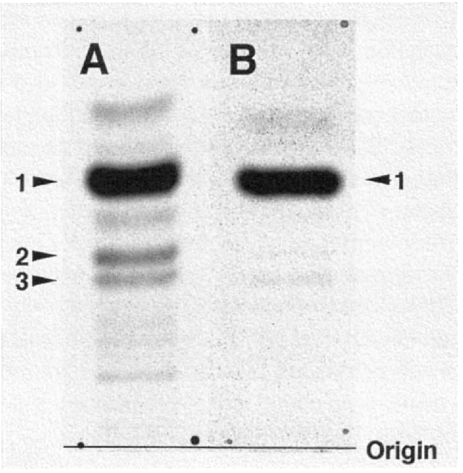

Fig. 2. Autoradiograms of steroid metabolites produced by medaka ovarian follicles incubated with [4-^{14}C] androstenedione. Ovarian follicle cells were collected at 18 hr (A) or 10 hr (B) before spawning. 1, androstenedione; 2, testosterone; 3, estradiol-17β.

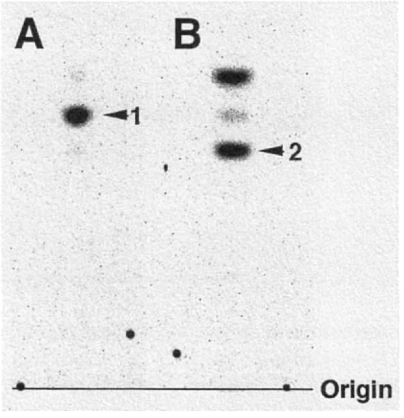

Fig. 3. Rechromatography of the metabolites 2 and 3 from Fig. 2. The two metabolites were scraped from TLC-I, benzene-acetone (4:1) and rechromatographed in TLC-II, cyclohexane-ethanol (9:1). A: 1, testosterone; B: 2, estradiol-17β.

follicles were further incubated with or without [1, 2-^{3}H]-17α-hydroxyprogesterone for 6 hr. The pattern of steroidogenesis was similar to that seen in controls (Fig. 4A, in the absence of PMSG) during the first 2 hr of incubation with PMSG (Fig. 4B). The major metabolites were androstenedione (metabolite 2) and a more polar metabolite. Incubation of intact follicles with PMSG for 12 hr (Fig. 4D) resulted in marked changes in the pattern of 17α-hydroxyprogesterone metabolites; the pattern was similar to that seen in maturing follicles incubated with 17α-hydroxyprogesterone (Fig. 1D). Although there was no obvious decrease in androstenedione production, a polar metabolite corresponding to 17α, 20β-DP was observed with several other polar metabolites.

Incubation of vitellogenic follicles with [4-^{14}C]-progesterone in the presence of IMBX alone and in combination with TPA and/or A23187

Effects of IBMX (a phosphodiesterase inhibitor) alone and in combination with TPA (a protein kinase C activator) and/or A23582 (a calcium ionophore) on progesterone metabolites were investigated using vitellogenic follicles collected at 18 hr before spawning. The follicles were incubated in media containing test compounds for 8 hr. After incubation, 1.00 × 10^{5} cpm of [4-^{14}C]-progesterone was added as a precursor. After further 3 hr of incubation, metabolites were extracted from incubation media and fractionated by two-dimensional TLC: benzene-acetone (4:1) in the first development system and cyclohexane-ethylacetate (1:1) in the second development system. IBMX (1 mM) stimulated androstenedione production (Fig. 5, column 2). This IBMX-stimulated androstenedione production was completely blocked by A23187 (5 mM) or TPA (0.5 mM) alone, or in combination with both compounds (Fig. 5, columns 3-6).

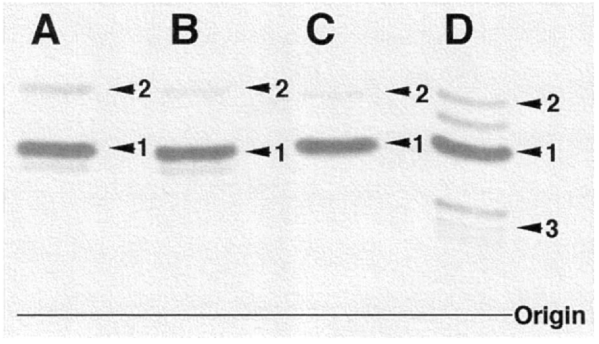

Fig. 4. Autoradiograms of steroid metabolites produced by medaka ovarian follicles incubated with [1, 2-^{3}H]-17α-hydroxyprogesterone in the absence (A, C) or presence (B, D) of PMSG. Two (A, B) and 12 (C, D) hr after the onset of incubations with PMSG, [4-^{14}C]-17α-hydroxyprogesterone was added. Six hours after the addition of the steroid, metabolites were extracted from media and applied to TLC fractionation. 1, 17α-hydroxyprogesterone; 2, androstenedione; 3, 17α, 20β-DP.

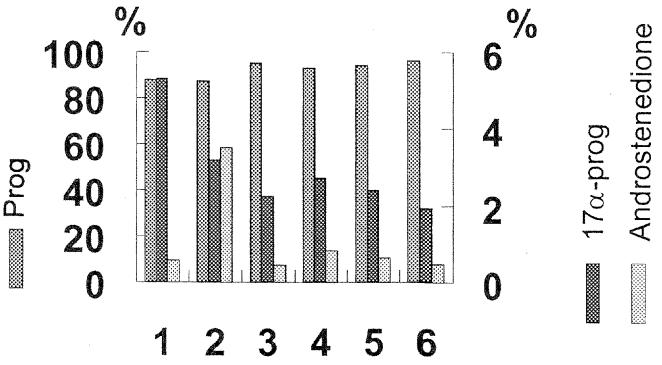

Fig. 5. Effects of IBMX, TPA, A23187 on progesterone metabolites by vitellogenic follicles collected 18 hr before spawning. Data are shown as percentage of recovered radioactivity. 1, control; 2, IBMX (1 mM); 3, IBMX (1 mM) + A23187 (5 mM); 4, IBMX (1mM) + TPA (0.5 mM); 5, IBMX (1 mM) + A23187 (5 mM) + TPA (0.1mM); 6, IBMX (1 mM) + A23187 (5 mM) + TPA (0.5 mM).

DISCUSSION

Data presented in this study indicate that there is a distinct shift in the steroidogenic pathway from estradiol-17β to 17α, 20β-DP by medaka ovarian follicles. It was shown that both estradiol-17β and 17α, 20β-DP are synthesized by the $^{5}\Delta$-steroid pathway (cholesterol → pregnenolone → 17α-hydroxy-pregnenolone → 17α-hydroxyprogesterone) (Fig. 6). Thus, in medaka ovarian follicles, the steroidogenic enzyme which serves as a branch point for estradiol-17β and 17α, 20β-DP production is cytochrome P-450 17α-hydroxylase/C17,20-lyase (P-450c17). P-450c17 is a single cytochrome P-450 enzyme mediating both 17α-hydroxylase and C17, 20-lyase activities (pig, Nakajin and Hall, 1981; Nakajin *et al.*, 1981, 1984; guinea pig, Tremblay *et al.*, 1994; bovine, Zuber *et al.*, 1987; rat, Namiki *et al.*, 1988; human, Yanase *et al.*, 1989; rainbow trout, Sakai *et al.*, 1992). Recently, we isolated a cDNA encoding P-450c17 from a medaka ovarian follicle cDNA library. The medaka P-450c17 expressed in non-steroidogenic COS-1 cells showed both 17α-hydroxylase and C17, 20-lyase activities (Kobayashi *et al.*, unpublished).

When medaka vitellogenic follicles were incubated with ^{3}H-labeled pregnenolone, the major metabolites were 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and androstenedione. By contrast, maturing follicles, under the same conditions, produce 17α-hydroxypregnenolone and 17α-hydroxyprogesterone. There is substantial reduction in the synthesis of androstenedione and, almost complete inhibition of testosterone and estradiol-17β production. Subsequently, there is an increase in the synthesis of 17α, 20β-DP. Furthermore, it was shown that the absence of androstenedione is not due to its rapid conversion to other metabolites. These results suggest that there is a distinct decrease in C17, 20-lyase activity of P-450c17 and this decrease is a critical mechanism that regulates the shift in the steroidogenic pathway.

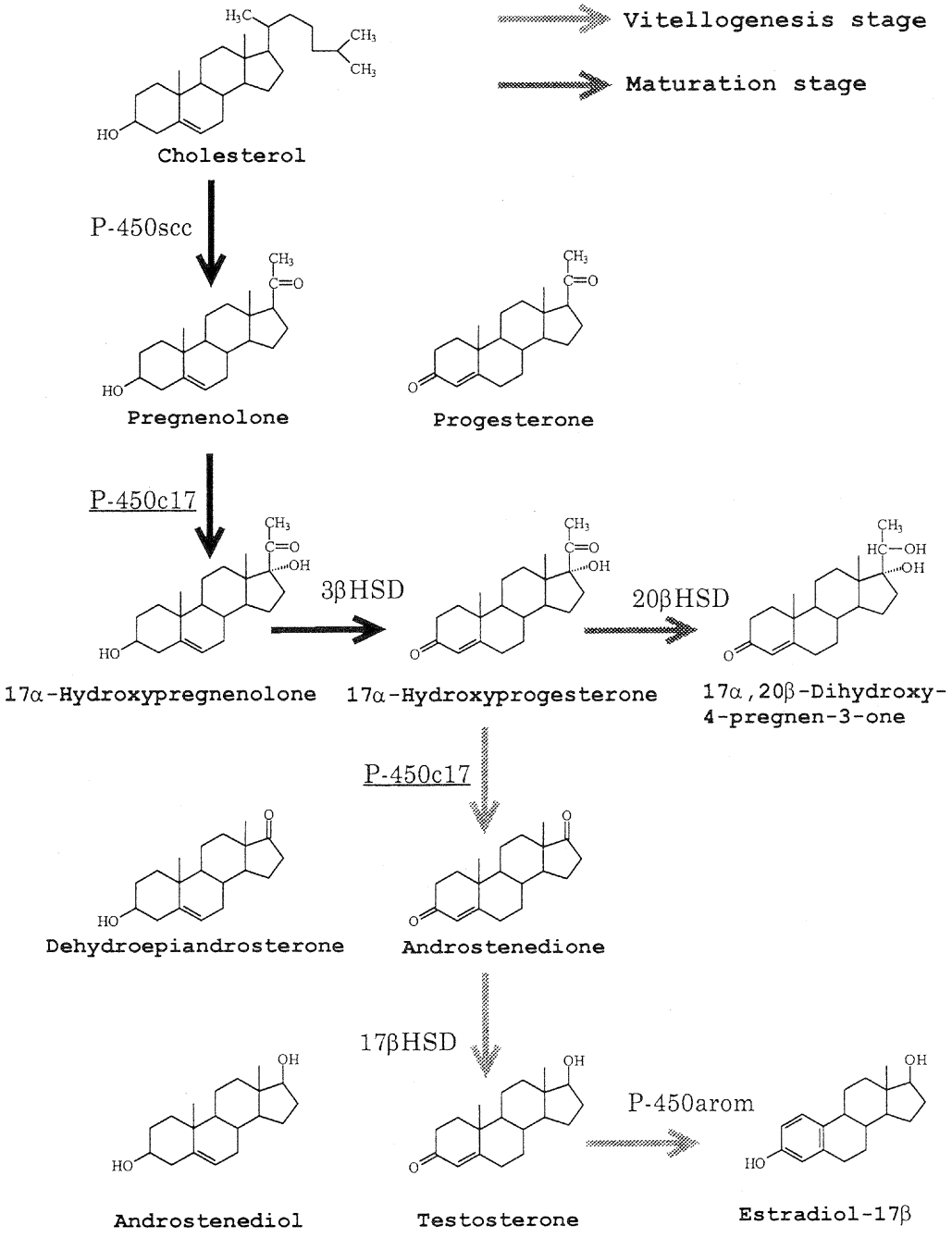

Fig. 6. Steroidogenic pathways of ovarian estradiol-17β and 17α, 20β-dihydroxy-4-pregnen-3-one biosynthesis by medaka ovarian follicles during vitellogenesis and oocyte maturation.

In mammals, inhibition of estradiol-17β production following the preovulatory LH surge is primarily the result of decrease in androgen production in theca and interstitial cells. In *in vitro* study with rat Grafian follicles in culture, inhibition of androgen secretion appeared to result from reduced 17α-hydroxylase and/or C17, 20-lyase activity (Gore-Langton and Armstrong, 1988). In the tropical catfish, *Clarias macrocephalus*, human chorionic gonadotropin (hCG) treatment *in vivo* induces an *in vitro* switch in ovarian steroidogenesis from predominantly testosterone to 17α, 20β-DP: a decrease in both 17α-hydroxylase and C17, 20-lyase activities of P-450c17 and a rapid increase in 20β-HSD activity (Suzuki *et al.*, 1989). It was hypothesized that the shift is regulated by the inhibition of C17, 20-lyase by gonadotropin (Scott and Baynes, 1982). However, our PMSG experiments using vitellogenic follicles collected at 22 hr before spawning demonstrated that gonadotropin is probably not directly responsible for the decrease in C17, 20-lyase activity in madeka ovarian follicles. The fish described by Suzuki *et al.* (1989) were post-ovulatory. The differences in the effect of gonadotropin on ovarian steroidogenesis

suggest that there may be major changes in steroidogenic activity over a very short time interval during the preovulatory phase. Further studies using larger numbers of fish at a more frequent sampling schedule are required to delineate more precisely the steroidogenic changes occurring during this period.

A very rapid steroidogenic pathway from androgen (11-ketotestosterone) to progestogen production has also been reported in several spermiating male fishes (Barry *et al.*, 1990). Thus, the differential regulation of 17α-hydroxylase and C17,20-lyase activities of P-450c17 is important for the process of final gamete maturation in both female and male fish. Barry *et al.* (1990), using cultures of testicular fragments of spawning male common carp, *Cyprinus carpio,* demonstrated that gonadotropin was not probably directly responsible for controlling the shift in the steroidogenic pathway from androgen to progestogen production. On the basis of data from Inano *et al.* (1967), Baynes and Scott (1985) postulated that the steroidogenic shift in rainbow trout may be regulated by the inhibition of C17, 20-lyase by 17α, 20β-DP. In fact, Barry *et al.* (1989) tested this hypothesis in common carp and demonstrated that at least one of the two enzymes that convert testosterone to 11-ketotestosterone, 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase, and probably C17, 20-lyase, are inhibited in a dose-dependent manner by physiological levels of 17α, 20β-DP. Certainly, this hypothesis merits further investigation using medaka ovarian follicles.

In this study, IBMX stimulated androstenedione production. It is of interest to note that A23187 and TPA supressed IBMX-induced androstenedione production. These results suggest that calcium and C-kinase regulate C17, 20-lyase activity in medaka ovarian follicles. In fact, bovine and human P-450c17 promoter regions contain not only cAMP responsive sequence (CRS) but also TPA responsive element (TRE). TPA was reported to suppress basal and cAMP stimulated transcription of P-450c17 through the TRE (Bakke and Lund, 1992; Brentano *et al.*, 1990). Thus, it is possible that in medaka ovarian follicles TRE also functions as a regulator of *P*-450c17 transcription in concert with CRS.

Another regulator of the enzymatic activities of P-450c17 is cytochrome b5. It has been reported that in reconstituted systems, the C17, 20-lyase activity in human P-450c17 is virtually undetectable in the absence of cytochrome b5. However, the addition of cytochrome b5 enhances C17, 20-lyase activity to a level much higher than 17α-hydroxylase activity (Katagiri *et al.*, 1995, Lee-Robichaud *et al.*, 1995). A similar enhancing effect of cytochrome b5 on the C17, 20-lyase activity is also seen in pig P-450c17 (Lee-Robichaud *et al.*, 1995). These results suggest that cytochrome b5 plays an important role in the differential regulation of the hydroxylase and lyase activities of P-450c17. Whether the level of cytochrome b5 influences the C17, 20-lyase activity in medaka ovarian follicles needs to be determined.

In summary, this study demonstrated that a distinct shift in the steroidogenic pathway from estradiol-17β to 17α, 20β-DP production occurs in medaka ovarian follicles prior to oocyte maturation. The inhibition of C17, 20-lyase activity may be directly responsible for controlling this shift. The inhibition of C17, 20-lyase would lead not only to a decrease in estradiol-17β production, but also to an accumulation of 17α-hydroxyprogesterone, the immediate precursor of 17α, 20β-DP, the maturation-inducing hormone of medaka. Fish ovarian follicles represent a useful model for future studies on the molecular mechanism of differential regulation of 17α-hydroxylase and C17, 20-lyase activities of P-450c17.

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